

Quantifying *Aphanomyces euteiches* in Alfalfa with a Fluorescent Polymerase Chain Reaction Assay

G. J. Vandemark, B. M. Barker, and M. A. Gritsenko

First author: Vegetable and Forage Crops Production Unit, U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS), Prosser, WA 99350; second author: Vegetable and Forage Crops Production Unit, USDA-ARS; and third author: Washington State University-IAREC, Prosser 99350.

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ABSTRACT

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A polymerase chain reaction (PCR) assay using a set of specific primers and a dual-labeled probe (TaqMan) was developed to quantify the amount of *Aphanomyces euteiches* DNA in alfalfa plants exhibiting varying levels of disease severity. The study included isolates of race 1 and race 2 of *A. euteiches*. The assay also discriminated between alfalfa populations for resistance based on analysis of DNA extracted from bulked plant samples. Analysis of individual plants and bulked plant

samples of standard check populations with both pathogen isolates resulted in Spearman rank correlations between pathogen DNA content and disease severity index ratings that were greater than 0.75 and highly significant ($P < 0.0005$). In experiments with a race 1 isolate, the amount of pathogen DNA present in the resistant check WAPH-1 was significantly less than in the susceptible check Saranac. In experiments with a race 2 isolate, the amount of pathogen DNA in the resistant check WAPH-5 was significantly less than in either of the susceptible checks, Saranac and WAPH-1. Discrimination between commercial cultivars based on quantitative PCR analysis of bulked plant samples was similar to classification based on visual assessment of disease severity.

The oomycete *Aphanomyces euteiches* Dreschs. causes severe root rot of several leguminous crops, including pea (*Pisum sativum* L.) (29), common bean (*Phaseolus vulgaris* L.) (30), and alfalfa (*Medicago sativa* L.) (6). Pathogenicity tests have identified at least seven pathotypes of *A. euteiches* that exhibit differential pathogenicity to specific legume species (12,16,30). Within the alfalfa pathotype of *A. euteiches*, strains have been identified that are differentially pathogenic toward different alfalfa cultivars or germ plasms (12). These strains are classified as either one of two races based on the disease response of established populations (cultivars and germ plasms) under standardized testing conditions (8).

Aphanomyces root rot of alfalfa has been reported in several different areas of the United States, including the states of Iowa, Kentucky, Ohio, and Wisconsin (6,27,31,35). Disease in alfalfa is characterized by stunted seedlings that have chlorotic cotyledons, damping-off, and poor stand establishment (9). No effective control for this disease of alfalfa is available. To minimize losses, the cultivation of resistant alfalfa cultivars is recommended, along with the avoidance of poorly drained and heavily infested fields (9). Although the impact of *A. euteiches* on alfalfa production has not been extensively characterized, Vincelli et al. (35) observed that alfalfa cultivars with resistance to *A. euteiches* exhibited significantly better seedling health, yield, and persistence than cultivars with low resistance or susceptibility when grown in naturally infested soils. Wiersma et al. (36) observed that alfalfa seedling vigor and forage yield was positively correlated with level of resistance to *A. euteiches* in tests conducted in naturally infested fields. Similarly, Wiersma et al. (37) observed, for alfalfa grown

under wet conditions in naturally infested fields, that cultivars with resistance to *A. euteiches* had significantly higher yields than susceptible cultivars.

Commercial alfalfa cultivars must be evaluated for reaction to *A. euteiches* using a standardized test protocol established by the North American Alfalfa Improvement Conference Committee on Standardized Tests for the resistance rating to be accepted by the National Alfalfa and Miscellaneous Legume Variety Review Board. The standard test uses a scale of 1 to 5 for evaluating disease severity, where 1 = healthy plant and 5 = dead plant (8). Plants scored as either a 1 or 2 (slight necrosis of roots and hypocotyls) are considered resistant (8). Resistance is rated according to a scale based on data from standard tests (1). Populations are considered to have high resistance (HR) if greater than 50% of the plants are resistant. Populations that have 31 to 50% resistant plants are rated as resistant (R). Only populations that have less than 6% resistant plants are rated susceptible (S). The genetic basis of resistance in alfalfa has not yet been determined, although resistance to *A. euteiches* in pea was observed to be quantitatively inherited (21). Heritability of resistance in pea is reported to be low (21), suggesting that environmental variance may contribute considerably to total variance for resistance (7). An additional contributor to variance for resistance within alfalfa populations may be the segregation of multiple alleles conditioning resistance in a tetraploid genome.

At the present, 71% (117/165) of the available certified alfalfa cultivars with a fall dormancy rating of 2 to 4 have at least a resistant (R) rating to *A. euteiches* race 1 (1). However, resistance to race 2 isolates has been demonstrated for very few certified cultivars (24). Race 2 isolates of the pathogen have been isolated from field soil samples from Idaho, Maryland, Minnesota, Mississippi, North Carolina, Tennessee, Virginia, and Wisconsin, and results of cultivar trials suggest that race 2 is also present in Iowa (12,24,27).

Developing alfalfa cultivars with resistance to *A. euteiches* is difficult due to several factors. These factors include potential

Corresponding author: G. J. Vandemark
E-mail address: gvandemark@pars.ars.usda.gov

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variation within and among evaluators in use of an integer scale for disease rating (8). The use of an integer scale for rating resistance phenotype limits the ability of the breeder to discriminate among the most resistant plants. A breeder may identify plants that, upon visual inspection, appear to be free of disease symptoms, which would be given disease severity scores of 1, but the breeder must select among these plants that appear phenotypically similar based on subjective evaluations of plant health.

Another difficulty encountered in the development of alfalfa cultivars with resistance to *A. euteiches* is variation in virulence among different isolates of the same race. Malvick and Grau (24) observed that commercial cultivars of alfalfa expressed different degrees of resistance to different isolates of both race 1 and 2. The standard test for evaluating resistance in alfalfa populations to *A. euteiches* requires that the percentage of resistant plants of the resistant and susceptible check population fall within acceptable ranges of 35 to 60% and 0 to 5%, respectively. Tests for which the results do not fall into these acceptable ranges are not considered to be valid. The use of the standard test with highly virulent isolates can be problematic, because it may be necessary to modify the number of zoospores used to infect each plant in order to realize acceptable ranges of the percentage of resistant plants for the check populations.

In even the most resistant alfalfa cultivars, the percentage of plants resistant to *A. euteiches* may not greatly exceed 50%. Malvick and Grau (24) found that, among four commercial alfalfa cultivars classified as HR to *A. euteiches* race 1 isolate MF-1, the average percentage of resistant plants was 47.7%. Among two commercial cultivars classified as being HR to *A. euteiches* race 2 isolate NC-1, the average percentage of resistant plants was 38.5% (24). These results suggest that considerable improvements can be made in levels of resistance to *A. euteiches* in alfalfa.

It may be possible to improve resistance levels by developing an alternative method for evaluating disease resistance that more precisely quantifies the amount of disease present in infected roots. Such a method would afford the breeder greater ability to discriminate between plants that appear to be phenotypically similar based on the visual assessment of disease severity. A more quantitative assay also might allow for valid comparisons among populations for levels of resistance to *A. euteiches* when results for check populations do not fall within acceptable ranges for the percentage of resistant plants required for the standard test for resistance (8). The standard test for evaluating resistance in alfalfa populations to *A. euteiches* requires that between 200 and 240 individual plants are scored for each population (8). A method that could evaluate resistance through an analysis of bulked plant samples would facilitate the process by eliminating the need for individually scoring hundreds of plants.

As an alternative to the visual assessment of disease severity for determining levels of resistance to *A. euteiches*, several investigations have attempted to directly quantify the amount of pathogen present in infected roots (18,20,25). The amount of *A. euteiches* present in infected roots has been estimated previously by microscopically counting the number of oospores in infected roots (25), but this method is very tedious and not amenable to the analysis of large numbers of plants. Kraft and Boge (18) developed a polyclonal antiserum to quantify the pathogen in infected pea roots and, more recently, the pathogen was quantified by analysis of specific fatty acids (20). The previous two methods have only been applied to the study of pea, and in each case only a single isolate of the pathogen was used as a source of inoculum.

The development of techniques for real-time detection of fluorescent-labeled DNA fragments amplified using polymerase chain reaction (PCR) may provide another approach for accurately determining the amount of *A. euteiches* in infected plants (13,14,22,23). A PCR-based method also may facilitate distinguishing resistant and susceptible populations based on PCR analysis of bulked plant samples. Real-time detection of PCR products (am-

plicons) employs a chemistry that uses the 5' nuclease activity of *Taq* polymerase (14). After an initial period, successive cycles of PCR result in the exponential synthesis of the amplicon, which can be quantified automatically using software that measures increase in fluorescence (32). For each sample, a cycle threshold (C_T) is calculated, which is defined as the initial cycle number at which an increase in fluorescence above a baseline can be detected (14). The C_T values are used to calculate DNA quantity in the samples based on C_T values calculated for standards containing known amounts of DNA. These techniques have been used successfully to detect and quantify several plant pathogens, including *Potato leafroll virus* (32), *Diaporthe phaseolorum* (38), *Phomopsis longicolla* (38), *Phytophthora infestans* (2), *Phytophthora citricola* (2), *Potato mop top virus* (26), and *Tobacco rattle virus* (26).

Our objectives were to (i) develop a real-time quantitative PCR assay to examine the relationship between disease severity and the amount of *A. euteiches* DNA detected in roots of infected alfalfa plants, (ii) employ the assay to discriminate between standard check alfalfa populations for resistance to *A. euteiches* by analysis of DNA extracted from bulked plant samples, and (iii) use the assay to discriminate between commercial alfalfa cultivars for resistance to *A. euteiches* by analysis of DNA extracted from bulked plant samples. This article describes the development of such an assay and demonstrates with both type isolates a significant positive correlation between disease severity and the amount of pathogen DNA detected in infected roots of both single plants and bulked plant samples. Applications of this assay for breeding programs and research on disease resistance are considered.

MATERIALS AND METHODS

Pathogen isolates. Two different isolates of *A. euteiches* were used in this study: *A. euteiches* MF-1, considered the type isolate of race 1, and *A. euteiches* NC-1, the type isolate of race 2 (8). The cultures were maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) at room temperature.

Plant materials. Three different standard check alfalfa populations were evaluated: cv. Saranac and the germ plasms WAPH-1 (11) and WAPH-5 (17). Saranac is the susceptible check in standard tests for evaluating resistance in alfalfa to race 1 of *A. euteiches* and WAPH-1 is the resistant check (8). Both Saranac and WAPH-1 are susceptible checks in standard tests for evaluating resistance in alfalfa to race 2 of *A. euteiches* and WAPH-5 is the resistant check (8).

Fifteen different commercial alfalfa cultivars were evaluated. Three cultivars were evaluated for each resistance rating class (1): 1 = high resistance (>50% resistant plants; Ranier, WL 232HQ, and Ultralac), 2 = resistance (31 to 50% resistant plants; Winterking, WL 325 HQ, and 5347 LH), 3 = moderate resistance (15 to 30% resistant plants; 5246, 54V54, and 54Q53), 4 = low resistance (6 to 14% resistant plants; 5454, WL 252 HQ, and 53V08), and 5 = susceptible (0 to 5% resistant plants; 57Q77, 5888, and 58N57) (fall dormancy).

Inoculations and evaluation of disease severity. The standard test protocol for evaluating resistance in alfalfa to *A. euteiches* (8,17) was followed in this study. Briefly, alfalfa seed were mixed in a commercial preparation of *Rhizobium meliloti* (LiphaTech Inc., Milwaukee, WI) and planted in plastic greenhouse flats containing vermiculite. Seedlings were grown in the greenhouse with 16-h day length at 20 to 24°C. Zoospores of each isolate of *A. euteiches* were produced in a mineral salt solution as described by Carman and Lockwood (5). At 1 week postgermination, the vermiculite was saturated with water and each seedling was inoculated with 1 ml of a 500 zoospore per ml of suspension by pipetting the suspension to the base of the stem. The flats were flooded for 5 days. Flats containing healthy seedlings were similarly flooded. Seven days after inoculation, all flats were drenched with a nutrient solution (Miracle-Gro; The Scotts Co., Columbus, OH).

Fourteen days after inoculation, disease severity index (DSI) ratings were done on the surviving seedlings using an integer scale from 1 to 4 as follows: 1 = no necrosis of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyl, slight chlorosis of cotyledons, and moderate stunting of stem; and 4 = extensive necrosis of roots, hypocotyls, and cotyledons, and severe stunting of stem (8).

Evaluation of individual plants. An experiment was conducted with each isolate of *A. euteiches* to examine the relationship between disease severity and the amount of pathogen DNA detected in roots of individual alfalfa plants. For experiments with *A. euteiches* MF-1 (race 1), flats were planted with two rows each of both the susceptible Saranac and resistant WAPH-1 checks. For experiments with race 2 isolate NC-1, flats were planted with two rows of each susceptible check (Saranac and WAPH-1) and the resistant check WAPH-5. Five days after sowing, the rows were thinned to a plant density of ≈ 50 seedlings per row. Inoculum was prepared and plants were inoculated as described above. Flats also were maintained in vermiculite that contained all three checks for a source of DNA from uninfected seedlings, which served as controls in quantitative PCR analysis. These flats were maintained on different greenhouse benches from the flats containing infected plants.

In the experiment with race 1 isolate MF-1, six plants of the resistant alfalfa check WAPH-1 were individually collected for each DSI class (1 to 4). In the experiment with race 2 isolate NC-1, six plants of the resistant alfalfa check WAPH-5 were individually collected for each DSI class. These experiments were conducted simultaneously, in the same greenhouse environment. Each experiment was repeated once. DNA was subsequently isolated from plants individually and analyzed by quantitative PCR.

Evaluation of bulked samples of standard checks. An experiment was conducted for each isolate of *A. euteiches* to examine the ability to discriminate check populations for resistance based on the analysis with quantitative PCR of DNA extracted from the roots of bulked plant samples. Experiments with *A. euteiches* MF-1 (race 1) used the susceptible Saranac and resistant WAPH-1 check populations, whereas experiments with race 2 isolate NC-1 used both susceptible check populations (Saranac and WAPH-1) and the resistant check WAPH-5. Inoculum was prepared and plants were inoculated as described above. Surviving seedlings were counted and scored for DSI as described above. For each population, the seedlings were randomly bulked into four groups of 15 plants. Uninoculated plants of each population also were bulked into groups of 15 plants. Experiments were conducted simultaneously, in the same greenhouse environment for both pathogen isolates. Each experiment was repeated once. DNA was subsequently isolated from each sample of bulked plants and analyzed by quantitative PCR.

Evaluation of bulked plant samples of commercial cultivars. An experiment was conducted with *A. euteiches* race 1 isolate MF-1 to examine the ability to discriminate commercial alfalfa cultivars for resistance based on the analysis with quantitative PCR of DNA extracted from the roots of bulked plant samples. Flats were planted with single rows of the resistant check population WAPH-1, the susceptible check population Saranac, and the 15 commercial cultivars listed above. The experiment consisted of six flats, with each flat having a single row of each cultivar and check population. Inoculum was prepared and plants were inoculated as described above. Surviving seedlings were counted and scored for DSI as described above. For each flat, the surviving seedlings were randomly bulked into a single group of 15 plants for each cultivar and check population. DNA was subsequently isolated from each sample of bulked plants and analyzed by quantitative PCR. The experiment was conducted simultaneously, in the same greenhouse environment for all six flats. The experiment was repeated once.

DNA extraction. To extract DNA from individual plants, the entire root system of each plant was first rinsed in tap water and blotted dry on sterile paper towels. DNA was extracted from the entire root sample using the Fast-DNA kit (BIO 101, Inc., Carlsbad, CA) according to manufacturer's recommendations. To extract DNA from bulked plant samples, the plants of each bulk were placed with their hypocotyls together and 200 mg of root tissue most proximal to the mass of hypocotyls was excised with a razor blade. DNA was extracted from the bulk sample using the Fast-DNA kit as described above. DNA also was isolated as previously described (34) from pure cultures of *A. euteiches* isolates MF-1, and NC-1 grown in potato dextrose broth (PDB; Difco Laboratories). DNA was quantified with a fluorometer (TD-700; Turner Designs, Inc., Sunnyvale, CA), and diluted to 20 ng/ μ l for use in quantitative PCR reactions.

PCR primer and probe design. Previously, we had designed a sequence characterized DNA marker that only was amplified in isolates of *A. euteiches* (34). A 1,332-bp DNA sequence (GenBank Accession No. AF228037) corresponding to this marker was analyzed using Primer Express software (Applied Biosystems, Foster City, CA) to identify candidate sequences for PCR primers and probes. This software selected primer sequences with melting temperature (T_m) values between 58 to 60°C, and an internal probe having a T_m value between 68 to 70°C. Candidate primer and probe sequences then were analyzed for adherence to several sequence motif constraints, such as the amount of cytosine relative to guanine and the avoidance of more than four consecutive identical nucleotides, prior to determining which combinations of primers and probes should be selected. Based on the results of this sequence analysis, the primers/probe set 136F-161T-211R (Table 1) was selected, which amplified a 76-bp fragment.

The primers and internal probe were commercially synthesized (Applied Biosystems). The 5' terminus of the probe 161T (TaqMan; Applied Biosystems) was labeled with the fluorochrome 6-carboxyfluorescein (FAM) and the 3' terminus labeled with the quencher dye tetra-methylcarboxyrhodamine (TAMRA). Primers were stored at -20°C as 90 μM stocks in double-distilled (dd) H_2O and the probe was stored, protected from light, at -20°C .

Quantitation of *A. euteiches* DNA in infected plants. DNA isolated from individual plants and from bulked plant samples, both infected and healthy, was analyzed using the primer/probe set 136F-161T-211R (Table 1). For each DNA sample, triplicate reactions were run in 50- μ l reactions containing 100 ng of DNA, 900 nM forward primer 136F, 900 nM reverse primer 211R, 100 nM probe 161T, 5 μ l of dd H_2O , and 25 μ l of 2 \times TaqMan Universal PCR Master Mix (Applied Biosystems). Amplifications and detection of fluorescence were done using a GeneAmp 5700 Sequence Detection System (Applied Biosystems). The thermocycling profile for all PCR reactions was the manufacturer's suggested default cycling profile, which consists of an initial cycle of 2 min at 50°C, then a single cycle of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The concentrations of primers and probes listed above were determined through a series of optimization reactions for which a matrix of different primers and probe concentrations was tested in replicated PCR reactions using purified genomic DNA of *A. euteiches* MF-1. Opti-

TABLE 1. Sequence of primers and probe used for the detection of *Aphanomyces euteiches* DNA

Primer/ probe ^y	Sequence 5' \rightarrow 3'	Position (bp) ^z	Amplicon (bp)
136F	GACTGCAATGTCGTCCTCAAGACTT	136–158	76
161T*	CAACCACCGAGCGAGCCGC	161–179	...
211R	ACAAAGCTGAGATGAAGAGATCGA	211–188	...

^y Asterisk indicates the TaqMan probe.

^z Base pair position in sequence of marker specific to *A. euteiches* (GenBank Accession No. AF228037).

mal forward and reverse primer concentrations were chosen that resulted in the maximum normalized reporter fluorescence (ΔR_N). The optimal TaqMan probe concentration was chosen that resulted in the minimal C_T . Each analysis also included three nontemplate control reactions, in which ddH₂O was substituted for DNA.

For the quantification of *A. euteiches* isolate MF-1 (race 1), standard curves were constructed by including triplicate reactions containing pure DNA of isolate MF-1 that spanned six different initial DNA quantities per reaction (0.1, 1.0, 10, 25, 50, and 100 ng). Triplicate reactions containing 100 ng per reaction of DNA isolated from uninfected Saranac and WAPH-1 also were included to confirm that the primer/probe sets selectively amplified DNA from the pathogen genome and not from plant DNA. For the quantification of *A. euteiches* isolate NC-1 (race 2), standard curves were constructed using purified pathogen DNA as described previously. Triplicate reactions containing 100 ng per reaction of DNA isolated from uninfected Saranac, WAPH-1, and WAPH-5 also were included to confirm that the primer/probe sets did not amplify DNA from healthy roots.

Data analysis. Data from the amplification of DNA samples from individual plants of different DSI classes (1 to 4) was subjected to an analysis of variance (ANOVA) and a pairwise *t* test using JMP Statistical Discovery Software (SAS Institute, Cary, NC) to determine if differences in the amount of detected pathogen DNA were significant ($P \leq 0.05$) between plants of each DSI class. For each experiment, the Spearman rank correlation (28) between the DSI score and amount of pathogen DNA was calculated using the mean of three replicate quantitative PCR reactions for each plant sample.

Experiments were conducted with check alfalfa populations only, and also with check populations and commercial cultivars, to evaluate the ability of the quantitative PCR assay to discriminate for resistance based on the analysis of DNA from the roots of bulked plant samples. Data from the amplification of DNA from bulked samples was subjected to an ANOVA and a pairwise *t* test to determine if differences in the amount of detected pathogen DNA were significant ($P \leq 0.05$). For each experiment, the Spearman rank correlation (28) was calculated between the mean DSI score for a sample of 15 bulked plants and the mean of three replicate quantitative PCR reactions for each bulk sample.

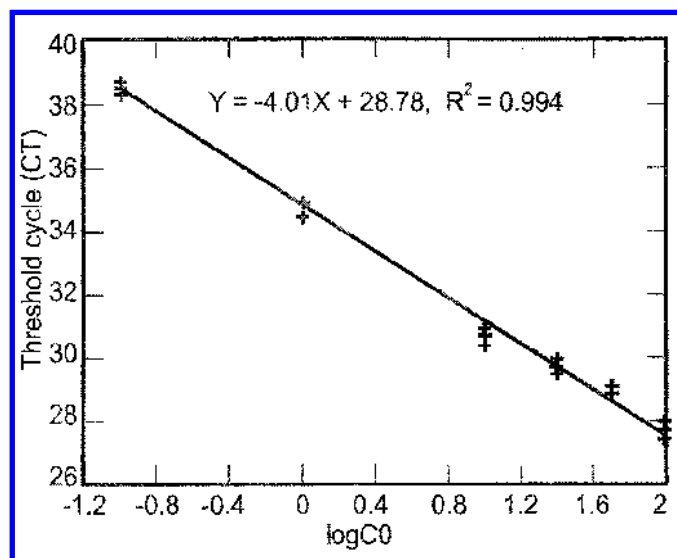


Fig. 1. Standard curve using known quantities (0.1, 1, 10, 25, 50, and 100 ng) of DNA from *Aphanomyces euteiches* isolate MF-1 amplified with the primer/probe set 136F-161T-211R. For each reaction, the cycle threshold (C_T), the initial cycle number at which an increase in fluorescence above a baseline can be detected, is plotted against the \log_{10} (Log CO) of the initial starting quantity of DNA. Three replicate polymerase chain reactions were performed for each quantity.

RESULTS

Primers/probe set sensitivity and specificity. For each experiment with a given pathogen isolate, standard curves were generated using DNA from that isolate. An example of a standard curve generated using DNA isolated from *A. euteiches* MF-1 and the primer/probe set 136F-161T-211R is presented (Fig. 1). The primer/probe set could detect DNA of both isolates in very linear assays within a range of 0.1 to 100 ng initial quantity. The results were very similar for both isolates, with the correlation between the \log_{10} of the initial DNA quantity and the C_T value ranging from -0.982 to -0.999 .

In all experiments, the nontemplate control reactions containing ddH₂O instead of DNA had C_T values equal to 40, indicating that no increase in fluorescence was detected. These results confirmed that the reagents were free of contaminating DNA. Experiments with race 1 isolate MF-1 also included reactions of DNA isolated from the roots of uninfected bulks of WAPH-1 and Saranac. Experiments with the race 2 isolate NC-1 included reactions for uninfected bulks of WAPH-1, Saranac, and WAPH-5. In all experiments, these samples also had C_T values equal to 40, confirming that the primer/probe set 136F-161T-211R did not amplify DNA from healthy plants.

Relationship between DSI and quantities of pathogen DNA detected in individual plants. In experiments with plants inoculated with race 1 isolate MF-1, ANOVA indicated that significant differences existed between DSI classes (1 to 4) for the amount of pathogen DNA detected in single plants ($P < 0.0001$). An example of the amplification plots obtained for single plants of each DSI class of WAPH-1 infected with isolate MF-1 is presented (Fig. 2). A comparison of means for the amount of pathogen DNA detected in plants of each DSI class is presented (Table 2). No significant difference in the amount of pathogen DNA detected was observed between DSI classes 1 and 2, whereas significantly less pathogen DNA was detected in DSI classes 1 and 2 than in DSI classes 3 and 4. Significantly more pathogen DNA was detected in plants of DSI class 4 than all other DSI classes.

The least amount of pathogen DNA detected in a 100-ng sample of total DNA isolated from the root of a single plant was 0.73 ng,

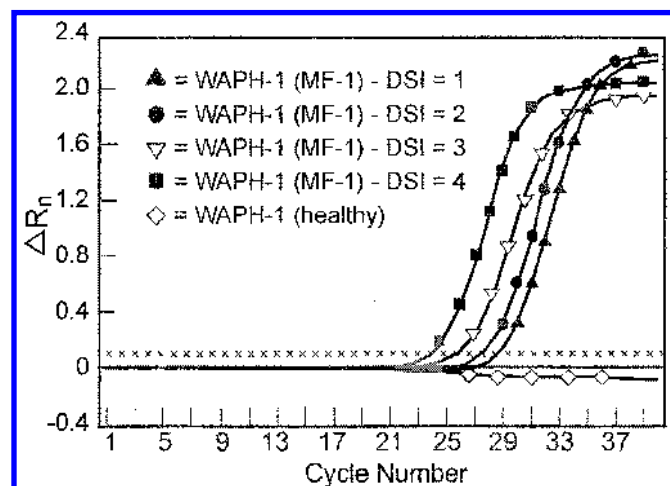


Fig. 2. Real-time amplification plot of total DNA isolated from roots of single plants of the alfalfa population WAPH-1 (resistant) infected with *Aphanomyces euteiches* isolate MF-1 and scored for disease severity index (DSI) ratings. DSI ratings correspond to the following resistance reactions: 1 = no necrosis of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyl, slight chlorosis of cotyledons, and moderate stunting of stem, and 4 = extensive necrosis of roots, hypocotyls, and cotyledons, and severe stunting of stem. Polymerase chain reaction (PCR) was performed using the primer set 136F-161T-211R. For each reaction, the fluorescent light emission (ΔR_N) is plotted versus the number of PCR cycles.

for a plant given a DSI score of 2. The largest amount of pathogen DNA detected was 21.33 ng, for a plant given a DSI score of 4. The Spearman rank correlation (Table 2) between DSI and the amount of *A. euteiches* race 1 isolate MF-1 DNA detected in individual plants was positive and significant.

In experiments with race 2 isolate NC-1, ANOVA indicated that significant differences existed between DSI classes (1 to 4) for the amount of pathogen DNA detected in single plants ($P < 0.0001$). A comparison of means for the amount of pathogen DNA detected in plants of each DSI class is presented (Table 2). A significant difference in the amount of pathogen DNA detected was observed between DSI classes 1 and 2. Significantly less pathogen DNA was detected in DSI classes 1 and 2 than in DSI classes 3 and 4. No significant difference in the amount of pathogen DNA detected was observed between DSI classes 3 and 4.

The least amount of *A. euteiches* NC-1 DNA detected in a 100-ng sample of total DNA isolated from the root of a single plant was 2.74 ng, for a plant given a DSI score of 1. The largest amount of pathogen DNA detected was 21.95 ng, for a plant given a DSI score of 3. The Spearman rank correlation (Table 2) between DSI and the amount of *A. euteiches* race 1 isolate MF-1 DNA detected in individual plants was positive and significant.

In this study, the entire root system of a sampled plant was used for DNA isolation. In all cases, even with the most susceptible plants sampled, for which only a very small and necrotic tap root was available, it was possible to extract approximately 100 μ l of a solution containing DNA at more than 20 ng/ μ l. This was sufficient DNA for at least 20 PCR reactions.

Discriminating between alfalfa standard check populations for resistance to *A. euteiches* races 1 and 2. ANOVA indicated that a significant difference was observed for the amount of DNA of *A. euteiches* race 1 isolate MF-1 detected in bulked plant samples of the resistant check WAPH-1 and the susceptible check Saranac ($P < 0.0001$). Significant differences were observed between experiments and significant effects attributable to population–experiment interactions also were observed ($P < 0.0001$). In an attempt to better understand the cause for this significant interaction, ANOVA was performed separately for both populations. Differences in the amount of MF-1 DNA detected in Saranac in both experiments were significant ($P < 0.0001$), and significant differences ($P = 0.0014$) also were observed for the amount of pathogen DNA detected in WAPH-1 in both experiments.

In both experiments, significantly ($P < 0.0001$) less MF-1 DNA was detected in the resistant check cv. WAPH-1 than in the sus-

ceptible check cv. Saranac. Results of comparisons of means between WAPH-1 and Saranac for the amount of pathogen DNA detected in roots of bulked samples are presented for a combined analysis of both replicate experiments (Table 3). The Spearman rank correlation between the mean DSI score for a bulk sample and the amount of *A. euteiches* MF-1 DNA detected in the bulk sample was positive and significant (Table 3).

ANOVA indicated that significant differences also were observed in the amount of DNA of *A. euteiches* race 2 isolate NC-1 detected in bulked plant samples of the three check populations ($P < 0.0001$). Differences between experiments and population–experiment interactions were not significant. In both experiments, significantly ($P < 0.0001$) less pathogen DNA was detected in the resistant check cv. WAPH-5 than in the two susceptible check populations. No significant differences in the amount of pathogen DNA detected were observed between the two susceptible check populations, Saranac and WAPH-1. Results of comparisons of means between check populations for the amount of pathogen DNA detected in roots of bulked samples are presented for a combined analysis of both experiments (Table 3). The Spearman rank correlation between the mean DSI score for a bulk sample and the amount of *A. euteiches* NC-1 DNA detected in the bulk sample was positive and significant (Table 3).

Discriminating between commercial alfalfa cultivars for resistance to *A. euteiches* race 1 type isolate MF-1. ANOVA indicated that significant differences were observed in the amount of pathogen DNA detected in bulked plant samples of the 17 tested populations ($P < 0.0001$). Differences between experiments and population–experiment interactions were not significant.

Results of comparisons of means between populations for the amount of pathogen DNA detected in roots of bulked samples are presented for a combined analysis of both replicate experiments (Table 4). The least amount of pathogen DNA was detected in the resistant check WAPH-1 and the greatest amount of pathogen DNA was detected in the susceptible check Saranac. The data based on quantitative PCR separated populations in a manner that was highly consistent with separation based on disease rating classes. The seven populations for which the lowest amounts of pathogen DNA were detected were the resistant check WAPH-1 and the six cultivars that were rated in either the class HR or R.

Data based on DSI ratings also separated populations in a manner that was highly consistent with separation based on disease rating classes. The seven populations with the lowest DSI means were the resistant check WAPH-1, five cultivars that were

TABLE 2. Comparison of means for quantity (ng) of *Aphanomyces euteiches* DNA in plants with different disease severity index (DSI) ratings of the resistant standard check alfalfa populations WAPH-1 and WAPH-5^y

DSI	WAPH-1	WAPH-5
	(<i>A. euteiches</i> MF-1)	(<i>A. euteiches</i> NC-1)
1	1.27 a	4.11 a
2	1.79 a	5.74 b
3	3.98 b	16.13 c
4	12.86 c	15.47 c
LSD ($\alpha = 0.05$) ^z	1.97	1.46
ρ (Prob > ρ)	0.85 (<0.0001)	0.83 (<0.0001)

^y Data is presented in each column for a combined analysis of two replicate experiments; $n = 12$ plants for each DSI rating. Means within a column followed by the same letter are not significantly different ($\alpha = 0.05$). DNA was extracted from roots of single plants and tested using the primer/probe set 136F-161T-211R. Each plant sample was tested with three replicate polymerase chain reactions. Ratings: 1 = no necrosis of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyl, slight chlorosis of cotyledons, and moderate stunting of stem, and 4 = extensive necrosis of roots, hypocotyls, and cotyledons, and severe stunting of stem. The Spearman rank correlation (ρ) between DSI and pathogen DNA quantity is presented.

^z LSD = least significant difference.

TABLE 3. Comparison of means between bulked plant samples of alfalfa standard check populations for disease severity index (DSI) ratings and quantity (ng) of *Aphanomyces euteiches* DNA^x

Population ^y	<i>A. euteiches</i> MF-1		<i>A. euteiches</i> NC-1	
	DNA (ng)	DSI	DNA (ng)	DSI
WAPH-1	2.12 a	2.79 a	13.58 a	3.99 a
Saranac	8.75 b	3.92 b	12.66 a	3.96 a
WAPH-5	2.63 b	2.66 b
LSD ($\alpha = 0.05$) ^z	1.60	0.13	1.23	0.13
ρ (Prob > ρ)	0.78 (0.0004)		0.79 (<0.0001)	

^x The Spearman rank correlations (ρ) between DSI and pathogen DNA quantity are presented. Means within a column followed by the same letter are not significantly different ($\alpha = 0.05$). Data in each column is for a combined analysis of two replicate experiments. Each experiment included four bulks of 15 plants each for each population. Prior to bulking, plants were individually scored using a DSI scale of 1 (healthy) to 4 (extensive necrosis of roots, hypocotyls, and cotyledons, and severe stunting of stem). DNA was extracted from roots of bulked plant samples and tested using the primer/probe set 136F-161T-211R. Each bulk DNA sample was tested with three replicate polymerase chain reactions.

^y *A. euteiches* MF-1: WAPH-1 = resistant; Saranac = susceptible. *A. euteiches* NC-1: WAPH-5 = resistant; WAPH-1 and Saranac = susceptible.

^z LSD = least significant difference.

rated in either the class HR or R, and a cultivar classified as having “moderate resistance” (Table 4). Saranac was tied with WL 252 HQ for the highest DSI mean. The Spearman rank correlation between the mean DSI score for a bulk sample and the amount of *A. euteiches* MF-1 DNA detected in the bulk sample was positive and significant (Table 4).

DISCUSSION

Previous examples of the application of real-time detection of fluorescent amplicons in plant pathology are limited (2,26,32,38). These examples primarily have focused on the use of this technology for the detection of plant pathogens in tubers (26,32), seeds (38), or individual plants (2). Here we report on the use of this technology for quantifying *A. euteiches* DNA in single plants and for discriminating among alfalfa populations for resistance to the pathogen.

Standard curves were generated for primer/probe set 136F-161T-211R with both *A. euteiches* isolate MF-1 and *A. euteiches* isolate NC-1, the type isolates for race 1 and race 2, respectively. Very high negative correlations between the initial DNA quantity and the C_T value were observed with both isolates (Fig. 1). This suggests that this approach for quantifying the amount of pathogen DNA in total DNA isolated from infected roots was precise. The observation that no amplicons could be detected in DNA isolated from the roots of healthy plants (Fig. 2) indicated that the primer/probe set selectively amplified a region of the pathogen genome.

TABLE 4. Comparison of means between bulked plant samples of commercial alfalfa varieties for disease severity index (DSI) ratings and quantity (ng) of DNA of *Aphanomyces euteiches* isolate MF-1 (race 1)^w

Population ^x	Resistance rating ^y	DNA (ng) ^z	DSI
WAPH-1	HR	1.08 (1) a	2.87 (3) ab
Winterking	R	2.25 (2) ab	2.70 (1) a
Ranier	HR	2.34 (3) ab	2.99 (6) b
WL 232HQ	HR	2.36 (4) ab	2.83 (2) ab
Ultralac	HR	2.71 (5) bc	2.88 (4) ab
WL 325HQ	R	3.72 (6) cd	2.98 (5) b
5347LH	R	3.79 (7) cd	3.49 (8) cd
5246	MR	4.14 (8) d	3.29 (7) c
54V54	MR	4.66 (9) de	3.68 (12) d-g
5454	LR	4.76 (10) d-f	3.72 (13) e-g
57Q77	S	4.84 (11) d-g	3.73 (14) e-g
54Q53	MR	5.60 (12) e-h	3.57 (9) de
5888	S	6.03 (13) f-i	3.82 (15) fg
WL 252 HQ	LR	6.07 (14) f-i	3.84 (16) g
58N57	S	6.08 (15) g-i	3.60 (10) d-f
53V08	MR	6.54 (16) hi	3.64 (8) d-g
Saranac	S	7.29 (17) i	3.84 (16) g
LSD ($\alpha = 0.05$)	...	1.31	0.22
ρ (Prob > ρ)	...	0.54 (<0.0001)	

^w Means within a column followed by the same letter are not significantly different ($\alpha = 0.05$). Number in parenthesis is the ranking among all tested populations ($n = 17$) for the trait. Data is presented in each column for a combined analysis of two replicate experiments. Each experiment included six bulks of 15 plants each for each population. Prior to bulking, plants were individually scored using a DSI scale of 1 (healthy) to 4 (extensive necrosis of roots, hypocotyls and cotyledons, and severe stunting of stem). Data is presented in each column for a combined analysis of two replicate experiments. The Spearman rank correlation (ρ) between DSI and pathogen DNA quantity is presented.

^x WAPH-1 = resistant, Saranac = susceptible, LSD = least significant difference.

^y HR = high resistance (>50% resistant plants), R = resistance (31 to 50% resistant plants), MR = moderate resistance (15 to 30% resistant plants), LR = low resistance (6 to 14% resistant plants), and S = susceptible (0 to 5% resistant plants) (1).

^z DNA was extracted from roots of bulked plant samples and tested using the primer/probe set 136F-161T-211R. Each bulk DNA sample was tested with three replicate polymerase chain reactions

The Spearman rank correlations between pathogen DNA content in single plants and DSI scores were positive and significant for both pathogen isolates (Table 3). These results indicate that plants that exhibited more resistance to the pathogen based on visual assessment of phenotype had less pathogen DNA in roots than more susceptible plants. This was likely the result of less pathogen growth and multiplication occurring in resistant plants. These results were similar to those obtained previously based on other methods to quantify the amount of *A. euteiches* present in pea lines (18,20,25). The counting of oospores in excised root tips indicated that a resistant pea line had lower numbers of oospores than a susceptible line (25). Kraft and Boge (18) demonstrated that more *A. euteiches* could be detected in homogenates from susceptible pea lines than from resistant lines based on enzyme-linked immunosorbent assay using a polyclonal antiserum that was specific to the pathogen. Recently, it was observed that the amounts of several fatty acids were positively correlated with the percentage of pea root systems containing *A. euteiches* oospores, but these experiments were conducted with only a single pea cultivar (20).

For experiments with *A. euteiches* isolate MF-1, no significant difference in pathogen DNA content was observed between plants of DSI classes 1 and 2, the two DSI classes considered to be resistant plants according to the standard test for resistance in alfalfa to *A. euteiches* (Table 3) (8). In this study, pathogen DNA was detected in all plants sampled. This suggests that none of the plants were immune to *A. euteiches*, although plants were identified that appeared to be highly resistant based on the visual assessment of disease severity. However, it is also possible that the detection of pathogen DNA in the roots of even the most resistant plants is the result of the assay detecting DNA from zoospores that encysted on roots but did not infect plants.

The highly positive and significant correlations between pathogen DNA quantity and DSI suggest it may be possible to use quantitative PCR to select for the most resistant plants from among a sample of plants that are considered to be equally resistant based on DSI rating. In this study, the entire root system of a sampled plant was used for DNA isolation. In all cases, for even the most susceptible plants sampled, sufficient DNA for at least 20 quantitative PCR reactions could be isolated from a single plant. It should be possible to take a root sample from the most resistant plants for DNA extraction, while maintaining enough of a root system to allow the selected plants to be replanted and maintained for use as breeding material. The utility of selection based on use of quantitative PCR would have to be evaluated by comparing the degree of population improvement observed using this strategy with that realized through selection based on visual assessment of resistance phenotype.

The results observed in this study suggest other possible applications of the use of quantitative PCR in the analysis of resistance to *A. euteiches*. It may be possible to use this approach to suggest different mechanisms of resistance to the pathogen. Low levels of pathogen DNA in resistant plants would characterize a mechanism that resulted in the inhibition of pathogen multiplication. An alternative mechanism might result in plants that were highly tolerant of infection by *A. euteiches*. These plants would be characterized by the presence of relatively high amounts of pathogen DNA in the root system coupled with a resistant phenotype.

We used this assay to discriminate between resistant and susceptible check populations based on the amount of pathogen DNA detected in randomly bulked plant samples. The success of this approach can be seen in that, in all experiments with both pathogen isolates, the resistant check had significantly less pathogen DNA than the susceptible check. Results obtained with WAPH-1, the resistant check for race 1 isolates of *A. euteiches* and a susceptible check for race 2 isolates of the pathogen, confirmed the validity of this approach. In experiments with *A. euteiches* race 1 isolate MF-1, significantly less pathogen DNA was detected in

WAPH-1 than was detected in the susceptible check Saranac (Tables 3 and 4). However, when challenged with *A. euteiches* race 2 isolate NC-1, the amount of pathogen DNA detected in WAPH-1 was not significantly different than in the other susceptible check, Saranac, yet both had significantly more pathogen DNA than the resistant check WAPH-5 (Table 3).

Significant differences were observed between experiments and significant effects attributable to population–experiment interactions were also observed for isolate MF-1. However, these results are consistent with previous observations that the severity of disease caused by *A. euteiches* is influenced by environmental factors encountered in controlled greenhouse environments, such as temperature (8,15,29). Nonetheless, in both experiments, significant differences were observed between Saranac and WAPH-1 for the amount of pathogen DNA detected by quantitative PCR.

In experiments with each isolate of *A. euteiches*, plants were individually scored for DSI prior to bulking. For both isolates, the Spearman rank correlation between the mean DSI for a bulk sample and the amount of pathogen DNA detected in the bulked sample was positive and significant (Table 3). These results indicate that in addition to the results observed for the analysis of single plants, a clear association between pathogen DNA quantity and disease resistance also was observed for bulked plant samples.

We also used this assay to discriminate between commercial alfalfa cultivars based on the amount of pathogen DNA detected in randomly bulked plant samples. Quantitative PCR separated populations in a manner that was highly consistent with separation based on disease rating classes (Table 4). The least amount of pathogen DNA was detected in the resistant check WAPH-1, whereas the greatest amount was detected in the susceptible check Saranac. The mean DSI rating for WAPH-1 was not significantly different from that of any of the three cultivars tested in the HR rating class, whereas Saranac was tied for the highest DSI mean. These results demonstrate that WAPH-1 and Saranac are appropriate resistant and susceptible standard checks for *A. euteiches* race 1 isolate MF-1. The Spearman rank correlation between the mean DSI score for a bulk sample and the quantity of *A. euteiches* MF-1 DNA in the bulk sample was positive and significant (Table 4). This result indicates that, in addition to the results observed for both the analysis of single plants and bulked plant samples of check populations, a clear association between the amount of pathogen DNA and disease resistance also was observed among bulked plant samples of many different commercial cultivars.

Alfalfa cultivars are almost exclusively synthetic populations that consist of seed resulting from the random mating of anywhere from ≈ 7 to more than 200 selected parental plants (4). As a consequence of such a breeding system, plants within cultivars vary for levels of resistance (4). An accurate assessment of the level of resistance to *A. euteiches* in a specific population has typically required that large (>200) numbers of plants be individually scored for resistance. The real-time quantitative PCR assay described here provided a reliable method for distinguishing among commercial alfalfa cultivars for resistance to *A. euteiches* based on the analysis of bulked plant samples.

Standard tests requiring the individual evaluation of many plants (≥ 200) must be conducted for determining levels of resistance in commercial alfalfa cultivars to other soilborne pathogens, including *Fusarium oxysporum* f. sp. *medicaginis* (3), *Verticillium albo-atrum* (10), *Phytophthora megasperma* (33), and the Columbia root knot nematode (*Meloidogyne chitwoodi*) (19). The evaluation of resistance to the Columbia root knot nematode is particularly tedious and time consuming, requiring the extraction of nematode eggs and their quantification by microscopy from between 300 and 500 individual plants (19). It may be possible to screen alfalfa populations for resistance to other pathogens by conducting quantitative PCR analysis of bulked plant samples. In addition to economic considerations regarding investments of time and resources associated with these assays, the utility of this

approach will be dependent upon two factors: (i) the availability of sequence information for developing primer/probe sets that selectively amplify pathogen DNA and do not amplify DNA from the genome of host plants and (ii) the ability to demonstrate a significantly positive correlation between pathogen DNA quantity and levels of disease resistance.

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